

# **EXHIBIT 12**

**NEW RECEPTOR  
AND RELATED PRODUCTS AND METHODS**

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This application is a continuation-in-part of co-pending application Serial No. 08/012,269 filed February 1, 1993, which is a continuation-in-part of co-pending application Serial No. 07/922,996 filed July 30, 1992, 10 which is a continuation-in-part of copending application Serial No. 07/267,577 filed November 7, 1988.

The subject matter described herein was in part a subject invention of NIH Grants Nos. IR23AI23058-03, R01 AI28175 and P60 KD20542 of which the present inventor was 15 the Principal Investigator and either the Donald Guthrie Foundation for Medical Research Inc. of Guthrie Square, Sayre, Pennsylvania 18849-1669 or Indiana University School of Medicine of Indianapolis, Indiana 46202, was the Grantee.

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**FIELD OF THE PRESENT INVENTION**

The present invention relates to a previously unknown 25 human receptor protein, H4-1BB, which was isolated and identified based upon work with a homologous murine (mouse) receptor protein, 4-1BB, which was isolated and identified by specific expression of the T cell genes by the present inventor.

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**BACKGROUND OF THE PRESENT INVENTION**

The immune system of humans and other species requires that white blood cells be made in the bone marrow, which 35 white blood cells include phagocytes, lymphocytes and B cells. As presently understood, the phagocytes include macrophage cells which scavenge unwanted materials such as

virus protein from the system. The lymphocytes include helper T cells and killer T cells and B cells as well as other cells, including those categorized as suppressor T cells. The B cells produce the antibodies. The killer T cells physically pierce the cell and the helper T cells facilitate the whole process. In any event, the immune process is facilitated by lymphokines.

Lymphokines are the proteins by which the immune cells communicate with each other. Scientists produce them in sufficient quantities for therapeutic use against immunologic diseases. There are many known lymphokine proteins and they include the interferons, interleukin-1,2,3,4,5,6,7, colony-stimulating factors, lymphotoxin, tumor necrosis factor and erythropoietin, as well as others.

Interleukin 1, secreted from macrophages activate the helper T cells and raise the body temperature causing fever which enhances the activity of the immune cells. The activated helper T Cells produce Interleukin 2 and Interleukin 2 stimulates the helper and killer T cells to grow and divide. The helper T cells also produce another lymphokine, B cell growth factor (BCGF), which causes B cells to multiply. As the number of B cells increases, the helper T cells produce another lymphokine known as the B cell differentiating factor (BCDF), which instructs some of the B cells to stop replicating and start producing antibodies. T cells also produce a lymphokine, gamma interferon (IF), which has multiple effects, like Interleukin 2. Interferon helps activate killer T cells, enabling them to attack the invading organisms. Like BCGF, interferon increases the ability of the B cells to produce antibodies. Interferon also affects the macrophages to keep them at the site of the infection and help the macrophages to digest the cells they have engulfed. Gathering momentum with each kind of lymphokine signal between the macrophages and the T cells, the lymphokines amplify the immune system response and the virus protein or

other foreign matter on the infected cells is overwhelmed. There are many other lymphokines, maybe a hundred or more, which participate in the immune process. Many lymphokines are known and many are not.

5       Lymphokines are sometimes called intercellular peptide signals. Among scientists there is widespread use of cloned cell lines as lymphokine producers and the isolation of lymphokine mRNA has become a common technique. The mouse receptor protein, 4-1BB, was isolated and identified based  
10 on specific expression of the T cell genes using a technique identified by the present inventor in a publication (Proc. Natl. Acad. Sci. USA. 84, 2896-2900, May 1987, Immunology). The protocol reported in this  
15 publication can be used by scientists to detect virtually all of the lymphokines. The method is designed to detect virtually all mRNA expressed differentially and the mRNA sequences of the immune cells are expressed differentially (as they relate to the T cells and the killer T cells) even though the level of expression is low and the quantity of  
20 the secreted lymphokine protein is low. The present inventor believes that the analysis described in the above identified publication can reveal biologically important molecules such as lymphokines because there are many indications that biologically important or active molecules  
25 are coded by the most scarce messages. An example is a transforming growth factor (TGF) which is present as only one of a million clones.

Most T cell factors have been classically identified by recognizing biologic activities in assays, purifying the  
30 protein information. An alternative approach is to isolate putative T cell genes based upon specific expression and then demonstrate the function of the unknown molecule. Using the aforesaid modified differential screening procedure, the present inventor cloned a series of T cell  
35 subset-specific cDNAs from cloned helper T (HTL) L2 and cloned cytolytic T lymphocyte (CTL) L3.

A series of T-cell subset-specific cDNAs were isolated from cloned murine T-cells by employing a modified differential screening procedure. The nucleotide sequence and expression properties of some of the cDNA species have  
5 been reported. One of the genes not previously characterized, that encodes mouse receptor protein 4-1BB, was studied further. These studies have led to the isolation of the human homologue to 4-1BB, H4-1BB.

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#### SUMMARY OF THE PRESENT INVENTION

The present invention includes the human receptor protein H4-1BB and the cDNA gene encoding for human  
15 receptor protein H4-1BB. The nucleotide sequence of the isolated cDNA is disclosed herein along with the deduced amino acid sequence. The cDNA gene identified as pH4-1BB was deposited at the Agricultural Research Service Culture Collection and assigned the accession number: NRRL B21131

20 The cDNA, and fragments and derivatives thereof, can be used as a probe to isolate DNA sequences encoding for proteins similar to the receptor protein encoded by the cDNA. The cDNA of the human receptor H4-1BB is about 65% homologous to the mouse cDNA 4-1BB and was isolated by  
25 using probes derived from cDNA 4-1BB. The cDNA gene identified as p4-1BB was deposited at the American Type Culture Collection at 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC No.: 67825.

The human receptor protein H4-1BB can be produced by:  
30 1) inserting the cDNA of H4-1BB into an appropriate expression vector, 2) transfecting the expression vector into an appropriate transfection host, c) growing the transfected hosts in appropriate culture media and d) purifying the receptor protein from the culture media. The  
35 protein and fragments and derivatives can be used: 1) as a probe to isolate ligands to human receptor protein H4-

1BB, 2) to stimulate proliferation of B-cells expressing H4-1BB ligands, or 3) to block H4-1BB ligand binding.

B-cell proliferation can be induced by treating B-cells that have expressed a ligand to receptor protein H4-1BB with cells that have expressed receptor protein H4-1BB. The use of H4-1BB to block H4-1BB ligand binding has practical application in the suppression of the immune system during organ transplantation. A similar costimulatory immune system pathway is being analyzed for this type of application. See "Mounting a Targeted Strike on Unwanted Immune Responses", Jon Cohen, Science, Vol. 257, 8-7-92; "Long Term Survival of Xenogeneic Pancreatic Islet Grafts Induced by CTLA4Ig", Lenschow et al, Science Vol. 257, 7-8-92; and "Immunosuppression in Vivo by a Soluble Form of the CTLA-4 T Cell Activation Molecule", Linsley et al, Science Vol. 257 7-8-92.

A monoclonal antibody against H4-1BB can be used to enhance T-cell proliferation by treating T-cells that have expressed receptor protein H4-1BB with the anti H4-1BB monoclonal antibody. Some tumors are potentially immunogenic but do not stimulate an effective anti-immune response in vivo. Tumors may be capable of delivering antigen-specific signals to T cells, but may not deliver the co-stimulatory signals necessary for full activation of T cells. Expression of the co-stimulatory ligand B7 on of melanoma cells was found to induce the rejection of a murine melanoma in vivo. ("Tumor Rejection After Direct Co-Stimulation of CD8<sup>+</sup> T Cells by B7-Transfected Melanoma Cells", Sarah E. Townsend and James P. Allison, Science Vol. 259, 1-5-93.) A monoclonal antibody against H4-1BB may be capable of the same effect as it is now known to induce T cell proliferation and activation.

A fusion protein for detecting cell membrane ligands to human receptor protein H4-1BB was developed. It comprises the extracellular portion of the receptor protein H4-1BB and a detection protein (alkaline phosphatase) bound to the portion of the receptor protein H4-1BB. The portion

of the receptor protein H4-1BB binds to the cell membrane ligands and binding can be detected by relative activity assays for the detection protein. The fusion protein is placed in the presence of a cell suspected to express the  
5 receptor protein H4-1BB. Then the cell is washed of any fusion protein not bound to the cell membrane ligands. Once the washed cells are placed in the presence of a substrate for the detection protein and the relative activity of the detection protein can be measured.

10 The primary object of the present invention is the identification of the new human receptor, H4-1BB as identified herein by its sequence.

Another object of the present invention is to teach a fusion protein comprising the extracellular portion of H4-  
15 1BB and a detection protein.

Still another object of the present invention is to teach methods of using the cDNA H4-1BB, the receptor protein H4-1BB, the monoclonal antibody and the legend for H4-1BB.

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#### BRIEF DESCRIPTIONS OF THE FIGURES

Figure 1 shows the sequence for the cDNA of mouse  
25 receptor protein 4-1BB and the regions used as PCR primers to obtain the human homologue H4-1BB.

Figures 2a and 2b show the nucleotide sequence and the deduced amino acid sequence of human receptor H4-1BB respectively.

30 Figures 3a and 3b illustrate the molecules involved in T-cell activation.

Figures 4a, 4b, and 4c illustrate a normal T-cell activation pathway.

Figures 5a, 5b, and 5c illustrate CTLA4-Ig alone, 4-  
35 1BB/AP and CTLA4-Ig together and 4-1BB/AP alone respectively being used to block steps in the T-cell activation pathway.

### DETAILED DESCRIPTION

In the following detailed description references are made to known procedures and studies, as well as published work of the applicant. These publications are incorporated herein by reference for clarity and listed in an appendix included at the end of this detailed description.

#### 10 Isolation and characterization of mouse receptor 4-1BB

Figure 1 shows the nucleotide sequence and the deduced amino acid sequence of the mouse receptor 4-1BB. The nucleotides of the message strand are numbered in the 5' to 3' direction and numbers are shown on both sides of the sequence. Nucleotide residue 1 is the A of the initiation codon ATG, and the nucleotides on the 5' side of residue 1 are indicated by negative numbers. The predicted amino acid sequence is shown below the nucleotide sequence. Putative signal peptide is underlined. Stop codon is indicated by (---). Cysteine residues are highlighted by the dots. An unusual feature of 4-1BB sequence is that there is a potential polyadenylation signal of AATAAA at nucleotides 1158-1163 (Fig. 1 boxed). It was believed that this signal was functional because this gene produces at least two different sizes of mRNA.

The transcript of 4-1BB was inducible by concanavalin A in mouse splenocytes, T-cell clones, and hybridomas. The expression of 4-1BB transcripts was inhibited by cyclosporin A. The 4-1BB mRNA was inducible by antigen receptor stimulation but was not inducible by IL-2 stimulation in the cloned T-cells (1). The 4-1BB cDNA encodes a peptide of 256 amino acids containing a putative leader sequence, a potential membrane anchor segment, and other features of known receptor proteins. Therefore, the expression pattern of 4-1BB resembles those of lymphokine mRNAs while the sequence appeared consistent with those of receptor proteins.



The major species of 4-1BB on the cell surface appears to be a 55-kDa dimer. 4-1BB also appears to exist as a 30-kDa monomer and possibly as a 110-kDa tetramer. Since these 4-1BB species were immunoprecipitated from a homogenous population of cells (T cell clone F1), all forms potentially co-exist on each cell. A comparison of peptide digests from the 4-1BB monomer and dimer will be needed to determine whether 4-1BB exists as a homodimer on the cell surface. A variety of cell surface receptors such as the insulin receptor (2), the B cell surface immunoglobulin receptor (3), the T cell Ag receptor (4), the CD28 costimulatory receptor (5), and the CD27 T cell antigen (6) are composed of disulfide-bonded subunits. Receptor dimerization may be required for ligand binding and subsequent biochemical signaling.

4-1BB is not expressed on resting T cells but is inducible by activators which deliver a complete growth stimulus to the T cell. The combination of PMA and ionomycin is capable of mimicing those signals required for T cell proliferation. Although PMA or ionomycin alone induced 4-1BB mRNA, the combination of PMA and ionomycin resulted in optimal 4-1BB expression. Furthermore, the expression of 4-1BB was not transient. When purified splenic T cells were stimulated with immobilized anti-CD3, 4-1BB mRNA was expressed and this expression was maintained for up to 96 hrs poststimulation. Cell cycle analysis will be required to confirm that 4-1BB is expressed throughout cell cycle progression.

4-1BB is structurally related to members of the nerve growth factor receptor super-family. Although these receptors possess structurally similar ligand-binding properties (cysteine-rich regions), the cytoplasmic domains of these proteins are nonconserved which could allow for diversity in transmembrane signaling. Some members of this family are involved in the T or B cell activation process. There are in vitro functional data on the OX-40, CD40 and CD27 antigens. Antibodies against the OX-40 augment the T

cell response in a mixed lymphocyte reaction (7) and antibodies against CD40 enhance B-cell proliferation in the presence of a coactivator, such as PMA or CD20 antibodies, and synergize with IL-4 in vitro to induce B-cell differentiation and to generate long-term normal B cell lines (8). One monoclonal antibody, anti-1A4, which recognizes an epitope on the CD27 molecule inhibited calcium mobilization, IL-2 secretion, helper T cell function, and T cell proliferation. On the other hand, CLB-CD27/1, another anti-CD27 mAb enhanced proliferation of human T cells stimulated with PHA or anti-CD3 mAb (6). These results indicate that the CD27 molecule plays an important role in T cell activation. Except for TNFRs, NCFR and CD40, the ligands or cell surface molecules to which the members of the superfamily bind are not yet identified. Identification and characterization of the ligands to which the receptors bind will be helpful in better defining the physiologic role of 4-1BB.

To ascertain whether cell surface 4-1BB could contribute to T cell activation, the anti-4-1BB 53A2 was used as an antagonist to 4-1BB. These data suggested that 4-1BB does in fact have the potential to function as an accessory signaling molecule during T cell activation and proliferation. The addition of soluble 53A2 to purified splenic T cells stimulated with immobilized anti-CD3 resulted in an amplification of <sup>3</sup>H thymidine incorporation compared to T cells stimulated with anti-CD3 alone. This pattern of enhancement ranged from 2- to 10- fold in three independent experiments.

In the original two signal model of Bretcher and Cohn, they proposed that signal 1, the occupancy of the T cell antigen receptor (TCR), resulted in inactivation of the T cell in the absence of signal 2, which is provided by accessory cells. This has since been confirmed by a variety of studies (9). The identification of the accessory cell CD28 as a potent costimulatory receptor on T cells was a significant contribution in beginning to characterize the

accessory signal(s) required for optimal T cell proliferation (10). It is possible that other cell surface molecules may contribute to these costimulatory activation requirements (11).

5       The biochemical signals delivered through 4-1BB are not completely known. One possibility considered was the observation that 4-1BB contains a putative p56<sup>lck</sup> tyrosine kinase binding domain in its cytoplasmic tail. It was later determined that p56<sup>lck</sup> tyrosinase kinase binds to  
10 4-1BB. It will also be worthwhile to determine if 4-1BB-mediated signaling can regulate genes such as IL-2 and IL-2 receptor, whose expression is required for T cell activation and subsequent proliferation.

Although the precise functions of members of the Nerve  
15 Growth Factor Receptor (NGFR) family appear to be diverse, an emerging theme is one in which these molecules may contribute in various ways to a maintenance of responsiveness or viability of the particular cell type in which they are expressed. For instance, NGF is absolutely  
20 required for viability of neurons in vitro and in vivo (12). The crosslinking of CD40 by soluble antiCD40 monoclonal antibody blocks germinal center centrocytes from undergoing apoptosis in vitro (13). Signals delivered  
25 through CD40 may also aid in maintenance of responsiveness to differentiation factors. The ligation of CD40 with anti-CD40 F(ab')<sub>2</sub> fragments in the presence of IL-4 induced large increases IgE synthesis (14). Also, anti-CD40 activated naive B cells treated with IL-10 and transforming growth factor- $\beta$  became committed to IgA secretion (15).

30       In addition to sharing the molecular characteristics with the NGFR superfamily, it was noted that the 4-1BB contained a putative zinc finger structure of the yeast eIF-2 $\beta$  protein (16). 4-1BB also shares a conserved region with the *sina* seven in absentia of *Drosophila*, which is  
35 required for correct photoreceptor cell development (17). That particular region is also similar to the protein

product of the DGl7 gene of *Dictyostelium*, whose expression is specifically induced during aggregation by cAMP (18).

This region forms the pattern of C-X<sub>2</sub>-C-X<sub>9</sub>-C-X<sub>3</sub>-H-X<sub>3</sub>-C-X-C; and the cysteines and histidine are  
5 conserved in a similar space in 4-1BB, *sina*, and DGl7 proteins. Ten of 24 amino acids between the 4-1BB and *sina* proteins are identical, and 3 of 24, are conservative substitutes. The conserved pattern suggests that these amino acids are functionally important. The *sina* protein is  
10 localized in the nucleus, suggesting that it has a regulatory function in cells. The fact that the amino acid sequence of 4-1BB contains features like a zinc finger motif, a nuclear protein, and a receptor domain suggests that 4-1BB may play diverse roles during cellular  
15 proliferation and differentiation.

4-1BB may represent another cell-surface molecule involved in T cell-APC interactions. The 4-1BB-AP fusion protein specifically bound to mature B-cell lines, anti- $\mu$ -activated primary B cells, and mature macrophage-cell  
20 lines. 4-1BB-AP bound at low or insignificant levels to immature B- and macrophage-cell lines, T-cell clones, T-cell lines, primary culture T cells, and various nonlymphoid-cell lines. Since 4-1BB-AP binds to mature B cells and macrophages, it is possible that signals  
25 delivered upon 4-1BB binding may modulate APC functions in some way. This possibility remains to be explored.

Chalupny and colleagues (19) have proposed that 4-1BB Rg, a fusion protein consisting of the extracellular domain of 4-1BB and the Fc region of human IgG, bound to the  
30 extracellular matrix (ECM). The highest level of 4-1BB Rg binding was to human vitronectin. In data not shown, an ELISA was performed using 4-1BB-AP and human vitronectin (Yelios Pharmaceuticals/GIBCO-BRL, Grand Island, NY.) immobilized at 0.007  $\mu$ g-10  $\mu$ g per well on microtiter  
35 plates. No binding of 4-1BB-AP based on AP activity was observed. To rule out the possibility that 4-1BB-AP was binding to proteins extrinsically attached to the cell

surface (possible extracellular matrix components), B-cell lymphomas were washed in acid conditions prior to the binding assay. 4-1BB-AP still bound specifically to mature B-cell lymphomas. It is still to be determined whether a 4-1BB-ligand specifically expressed on B cells and macrophages exists, and whether 4-1BB-AP may bind to the ECM under particular binding conditions. It is possible that the ECM could facilitate the binding of 4-1BB to a specific cell-surface ligand.

10 B cells and helper T cells interact with each other through receptors on B cells binding to their specific counter-receptors on T cells. It is thought that this interaction results in a cascade of biochemical signaling relays between these two cell types (20). As this  
15 interaction proceeds, these cells become committed to enter the S phase of the cell cycle. Initial interactions between TCR and CD4 on T cells, and processed antigen-MHC II on B cells, do not result in B cells capable of entering the cell cycle (21). However, studies from in vitro systems  
20 suggest that once T-cells are stimulated, they express newly synthesized or modified cell-surface molecules capable of inducing B cells to enter the cell cycle (22, 23). This T-cell function is not antigen-specific or MHC-restricted (24). In addition, soluble factors are not  
25 required for the activated Th induction of B-cell activation (25). Once B cells enter the cell cycle, IL-4 induces B cells to progress from G<sub>1</sub> to S phase. The ability of activated T cells or T-cell membranes to promote the entry of B cells into the cell cycle can be blocked by  
30 either cycloheximide or cyclosporin A treatment (26, 27). These newly expressed membrane proteins appear to be "lymphokine-like" in their induction characteristics.

4-1BB has expression properties which meet the requirements of a B-cell costimulator. 4-1BB is inducible  
35 by anti-CD3 or TCR-mediated T-cell stimulation, and its expression is sensitive to cyclosporin A as well as cycloheximide treatment (28). Interestingly,

paraformaldehyde-fixed SF21-4-1BB cells, synergized with anti- $\mu$  in inducing B-cell proliferation. The costimulation of splenic B cells by SF21-4-1BB occurred at optimal (10  $\mu$ g/ml) and suboptimal (1.0-0.1  $\mu$ g/ml) doses of anti- $\mu$ . The  
5 addition of SF21-4-1BB cells to resting B cells, did not result in significant B-cell proliferation. SF21-4-1BB cells did not synergize with TPA or ionomycin, or suboptimal concentrations of LPS in inducing B-cell proliferation.

10 Although the baculovirus system has been used to express large amounts of recombinant soluble proteins, this system may be utilized for the expression of recombinant cell-surface proteins. The baculovirus infection provides a convenient means to express uniformity high levels of  
15 recombinant protein on a per cell basis. It is noteworthy, that the addition of SF21 cells alone did not result in significant levels of costimulation. This can be a potential problem when using cos- or L- cell lines which can exhibit strong costimulator activity on their own.

20 Another member of the NGFR superfamily, CD40, is expressed on B cells and interacts with gp39, a molecule expressed on activated T cells. The cDNAs encoding the murine (29) and human (30) gp39 proteins have been cloned; this cell surface molecule is a type II membrane protein  
25 with homology to tumor necrosis factor. Noelle et al. (31) found that a CD40-inununoglobulin fusion protein, is capable of blocking T cell-induced B-cell proliferation and differentiation in a dose-dependent manner. Armitage et al. have isolated a cDNA for murine gp39 and showed that  
30 gp39 could induce B-cell proliferation in the absence of co-stimuli, and result in IgE production in the presence of IL-4-. Hollenbaugh et al. (32) have shown that COS cells transfected with human gp 39 can synergize with either TPA or anti-CD20 in inducing human B-cell proliferation and is  
35 able to stimulate B cells without a costimulator only at low levels. These data indicate that CD40 may be one of the

B-cell-surface molecules that transmit signals during physical contact with T cells.

Cell-surface receptors communicate with their external milieu by interacting either with soluble factors or other cell surface molecules expressed on neighboring cells. The role of biochemical signals delivered by cell-cell contact versus those delivered by soluble factors interacting with cell surface receptors is not clear. The NGFR superfamily is unusual for the TNFR I and II as well as the NGFR bind to more than one ligand. The TNFRs I and II both bind to TNF- $\alpha$  and TNF-R (33). The NGFR binds to NGF, brain-derived neurotrophic factor, and neurotrophin-3 (34).

In addition, one ligand may function as both a cell surface and soluble ligand. Recent evidence on the CD4-0 ligand, gp39, suggests that this ligand can exist as a membrane bound as well as a soluble ligand (35). It may be possible that 4-1BB is secreted and interacts with B cells in a soluble form as well as a membrane bound form. A member of the NGFR receptor family, CD27, which is expressed on T cells, is secreted in addition to being expressed on the cell surface (36). It is also possible that more than one 1 ligand (soluble and cell surface) may bind to 4-1BB.

#### 25 Isolation of the human homologue, H4-1BB

In order to isolate the human homologue (H4-1BB) of mouse 4-1BB two sets of polymerase chain reaction (PCR) primers were designed. To design the PCR primers, the amino acid sequence among the members of nerve growth factor receptor (NGFR) superfamily were compared because 4-1BB is a member of the superfamily (37). The amino acid sequences employed were mouse 4-1BB (38), human NGFR (39), human tumor necrosis factor receptors (33), human CD40 (40), and human CD27 (6). The areas of sequence conservation among the NGFR superfamily were chosen.

Forward primer I (H4-1BBFI) spans from amino acids 36 to 41 and forward primer II (HR-1BBFII) spans from amino

acids 52 to 58 of the mouse 4-1BB. Reverse primer I (H4-1BBRI) spans from amino acids 116 to 121 and reverse primer II (H4-1BBRII) spans from amino acids 122 to 128 of mouse 4-1BB. The regions used as PCR primers in mouse 4-1BB are indicated in Fig. 1.

The degenerative oligonucleotide sequence of each primer is as follows:

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1  H4-1BBFI:      5' TTC TGT CGI AAA TAT AAT CC 3'
                      T  C A      G  C  C
10  H4-1BBFII:     5' TTC TCI TCI ATT GGI GGI CA 3'
                      T  G  G  C
                      A
15  H4-1BBRI:      5' CC  IAA IGA ACA IGT TTT ACA 3'
                      G  CT G      C  G
    H4-1BBRII:     5' TT  TTG ATC ATT AAA IGT ICC 3'
                      C  G  G  G
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Peripheral blood lymphocytes from normal healthy individuals were isolated and activated with PMA (10 ng/ml) and ionomycin (1  $\mu$ M). mRNA from the lymphocytes was isolated. Using reverse transcriptase the human lymphocyte mRNA was converted to single-stranded cDNA. The cDNA was then amplified with Taq polymerase with combination of the primers. The combination of primers was as follows: H4-1BBFI vs H4-1BBRI; H4-1BBFI vs H4-1BBRII; H4-1BBFII vs H4-1BBRI; and H4-1BBFII vs H4-1BBRII.

The primer set of H4-1BBFII and H4-1BBRII produced a specific band of ~240bp. The 240bp is an expected size of human 4-1BB if the human homologue protein is similar to mouse 4-1BB in size. The PCR product (240bp) was cloned in PGEM3 vector and sequenced. One open reading frame of the PCR product was ~65% identical to mouse 4-1BB. Therefore, it was concluded that the 240 bp PCR product is the human homologue of mouse 4-1BB. The 240 bp PCR product was used to screen  $\lambda$ gt11 cDNA library of activated human T lymphocytes. An ~0.85 kb cDNA was isolated. The sequence of the cDNA is shown in Figure 2 and the predicted amino acid sequence is shown in Figure 2b. The same information



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is shown is the sequence listing attached to this specification in sequence id. 1.

An expression plasmid to produce H4-1BB-AP fusion  
5 protein was constructed. The 5' portion of the H4-1BB cDNA including sequences encoding the signal sequence and the entire extracellular domain, was amplified by PCR. For correctly oriented cloning, a Hind III site on the 5' end of the forward primer and a Bgl II site on the 5' end of  
10 the reverse primer were created.

The Hind III - Bgl II H4-1BB fragment was inserted into the mammalian expression vector APTaq-1, upstream of the coding sequence for human placental alkaline phosphatase (AP). The oligonucleotides PCR primers used  
15 for the amplification of 5' portion of H4-1BB are as follows:

Forward

primer: 5' AAT AAG CTT TGC TAG TAT CAT ACC T 3'

20 Reverse

primer: 5' TTA AGA TCT CTG CGG AGA GTG TCC TGG CTC 3'

H4-1BB-AP will be used to identify cells and tissues that express ligand for human 4-1BB (i.e. H4-1BBL). The  
25 studies with mouse 4-1BB indicated that the ligand for 4-1BB is on the cell surface. B cells and macrophages were major cells that express 4-1BBL. It is expected that H4-1BBL also expresses on human B cells and macrophages.

30 A mammalian expression cDNA library will be generated from human cell lines that express H4-1BBL. The library will be screened by [<sup>125</sup>] I-labeled H4-1BB-AP. cDNA for H4-1BBL will then be isolated and characterized. Soluble recombinant H4-1BBL will then be produced. Both H4-1BB-AP  
35 and H4-1BBL will be used to suppress or enhance immune responses as described below. Monoclonal antibody to H4-1BB and H4-1BBL will be produced.

According to studies with mouse 4-1BB, 4-1BB acts as a costimulatory signal. It is expected that H4-1BB will

act as a costimulatory signal for T cell activation. Mouse 4-1BB helped B cells with proliferation and differentiation. It is expected that H4-1BB will do the same. H4-1BB-AP, H4-1BBL and monoclonal antibody can be  
5 used to suppress or enhance human immune responses.

Figures 3a and 3b illustrate the molecules involved in T-cell activation. During early T-cell activation (cognitive phase), resting T cells express the TCR/CD3 complex and other "accessory" molecules. Among these  
10 constitutively expressed molecules, CD4 (or CD8), LFA-1 and CD28 are probably the ones to receive costimulatory signals. Initial interaction with the TCR/CD3 complex in combination with these 'accessory' costimulatory signals leads to subsequent expression of additional receptor  
15 molecules such as CD28, CTLA4, and 4-1BB. These newly expressed molecules are probably going to receive additional important costimulatory signals at later stages of T-cell activation (clonal expansion).

## 20 Suppression of immune responses.

Figures 4a-c illustrate a normal T-cell activation pathway. Figures 5a-c illustrate the blocking of immune responses with soluble chimera of 4-1BB. If 4-1BB plays a role in T-cell activation, blocking of the interaction to  
25 its ligand on antigen-presenting cells should result in suppression of T-cell dependent immune responses. It is well documented that blocking of the interaction of CD28 to its counter-receptor B7 suppresses in varying degrees, both in vivo antibody production and cell-mediated immune  
30 responses. Blocking of both interactions should result in a more effective immunosuppression; since 4-1BB is induced during T-cell activation. Blocking of the interaction of 4-1BB to its ligand may be of importance at later stages of the activation process where the CD28/B7 interaction may no  
35 longer be of relevance.

As illustrated with mouse receptor 4-1BB and mouse ligand 4-1BBL above, addition of H4-1BB-AP will coat the H4-

1BBL expressing cells and block the normal interaction between H4-1BB and H4-1BBL. This will lead to immunosuppression. This type of immunosuppression is antigen-specific. Therefore it avoids the generalized immunosuppression produced by antiCD3 or cyclosporin A treatments. H4-1BB-AP treatment can be used to treat certain autoimmune diseases and to facilitate organ transplantation.

10 Immune enhancement.

H4-1BB may function at the late stage of T cell activation and may be a critical molecule for completion of T cell activation. Most tumors display tumor-specific antigens. One reason, however, why immunogenic tumors can escape host immunity is that tumor-reactive T cells receive inadequate costimulation. The introduction of the costimulatory molecules, such as H4-1BB into the tumor, therefore, could enhance the antitumor immunity of cytotoxic T cells (CTL). H4-1BBL can be expressed in cell-specific fashion. For example, the H4-1BBL can be expressed in melanoma using melanocyte-specific promoter such as tyrosinase promoters. The H4-1BBL-expressing melanoma will stimulate cytotoxic T cells through H4-1BB and activate the melanoma-specific CTL. The activated melanoma-specific CTL can destroy melanoma.

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The foregoing description has been directed to particular embodiments of the invention in accordance with the requirements of the Patent Statutes for the purposes of illustration and explanation. It will be apparent, 5 however, to those skilled in this art that many modifications and changes will be possible without departure from the scope and spirit of the invention. It is intended that the following claims be interpreted to embrace all such modifications.

I claim:

1. A cDNA encoding for human receptor protein H4-1BB.
- 5 2. The cDNA of claim 1 having a nucleotide sequence as shown in Figure 2.
3. The cDNA of claim 1, identified as pH4-1BB deposited at the Agricultural Research Service Culture Collection with  
10 the accession number NRRL B21131.
4. The cDNA of claim 2 and fragments and derivatives thereof, wherein said fragments and derivatives can be used as a probe to isolate DNA sequences encoding for proteins  
15 similar to the receptor protein encoded by said cDNA.
5. The receptor protein H4-1BB produced by
  - a) inserting the cDNA of H4-1BB into an appropriate expression vector,
  - 20 b) transfecting said expression vector into an appropriate transfection host,
  - c) growing said transfected hosts in appropriate culture media and
  - d) purifying the receptor protein from said culture  
25 media.
6. A protein having the amino acid sequence shown in Figure 2.
- 30 7. The protein of claim 6 and fragments and derivatives thereof, wherein said fragments and derivatives:
  - a) can be used as a probe to identify ligands to receptor protein H4-1BB;
  - b) can be used to stimulate proliferation B-cell's  
35 expressing H4-1BB ligands; or
  - c) can be used to block H4-1BB ligand binding.



8. A monoclonal antibody against H4-1BB which specifically recognizes receptor protein H4-1BB.
9. A hybridoma capable of producing a monoclonal antibody  
5 against H4-1BB which specifically recognizes receptor protein H4-1BB.
10. The method of using the monoclonal antibody of claim 8 to enhance T-cell proliferation comprising the step of  
10 treating T-cells that have expressed receptor protein H4-1BB with said monoclonal antibody.
11. The method of claim 12 further comprising the step of conducting said treatment in the presence of protein  
15 tyrosinase kinase.
12. The method of using the monoclonal antibody of claim 8 to enhance T-cell activation comprising the step of treating T-cells that have expressed receptor protein H4-  
20 1BB with said monoclonal antibody.
13. The method of claim 12 further comprising the step of conducting said treatment in the presence of protein tyrosinase kinase.
- 25 14. A fusion protein for detecting cell membrane ligands to human receptor protein H4-1BB, comprising:
- a) at least a portion of said receptor protein H4-1BB corresponding to the extracellular portion of said  
30 receptor protein H4-1BB such that said portion of said receptor protein H4-1BB binds to said cell membrane ligands; and
- b) a detection protein bound to said portion of said receptor protein H4-1BB such that ligand binding can  
35 be detected by relative activity assays for said detection protein.

15. The fusion protein of claim 14 wherein said detection protein is alkaline phosphatase.

16. A method of detecting cell membrane ligands to human  
5 receptor protein H4-1BB, comprising:

a) providing a fusion protein including:

10 1) at least a portion of said receptor protein H4-1BB corresponding to the extracellular portion of said receptor protein H4-1BB such that said portion of said receptor protein H4-1BB binds to said cell membrane ligands, and

15 2) a detection protein bound to said portion of said receptor protein H4-1BB such that ligand binding can be detected by relative activity assays for said detection protein;

b) placing said fusion protein in the presence of a cell suspected to express said receptor protein H4-1BB;

20 c) washing said cell of any fusion protein not bound to said cell membrane ligands;

d) placing said washed cells in the presence of a substrate for said detection protein and measuring the relative activity of said detection protein.

25 17. The method of claim 16 wherein said detection protein is alkaline phosphatase.

18. A method of inducing B-cell proliferation comprising the step of treating B-cells that have expressed a ligand  
30 to human receptor protein H4-1BB with cells that have expressed receptor protein H4-1BB.

## ABSTRACT OF THE DISCLOSURE

5       The human receptor H4-1BB has been isolated, sequenced  
and disclosed herein. The cDNA of the human receptor H4-  
1BB is about 65% homologous to the mouse cDNA 4-1BB and was  
isolated by using probes derived from cDNA 4-1BB. A fusion  
protein for detecting cell membrane ligands to human  
10 receptor protein H4-1BB was developed. It comprises the  
extracellular portion of the receptor protein H4-1BB and a  
detection protein (alkaline phosphatase) bound to the  
portion of the receptor protein H4-1BB. B-cells that have  
expressed a ligand to receptor protein H4-1BB can be  
15 treated with cells that have expressed receptor protein H4-  
1BB and B-cell proliferation may be induced. The use of  
H4-1BB to block H4-1BB ligand binding has practical  
application in the suppression of the immune system during  
organ transplantation. A monoclonal antibody against H4-  
20 1BB can be used to enhance T-cell proliferation by treating  
T-cells that have expressed receptor protein H4-1BB with  
the anti H4-1BB monoclonal antibody. Tumors transfected  
with H4-1BBL may be capable of delivering antigen-specific  
signals as well as the co-stimulatory signals and can be  
25 killed by human cytotoxic T lymphocytes.

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Fig. 1

ATGTCCATGA	ACTGCTGAGT	GGATAAACAG	CACGGGATAT	CTCTGTCTAA	- 96										
AGGAATATTA	CTACACCAGG	AAAAGGACAC	ATTCGACAAC	AGGAAAGGAG	- 46										
CCTGTCACAG	AAAACCACAG	TGTCCTGTGC	ATGTGACATT	TCGCC	- 1										
ATG	GGA	AAC	AAC	TGT	TAC	AAC	GTG	GTG	GTC	ATT	GTG	CTG	CTG	CTA	45
Mbt	Gly	Asn	Asn	Cys	Tyr	Asn	Val	Val	Val	Ile	Val	Leu	Leu	Leu	
GTG	GGC	TGT	GAG	AAG	GTG	GGA	GCC	GTG	CAG	AAC	TCC	TGT	GAT	AAC	90
Val	Gly	Cys	Glu	Lys	Val	Gly	Ala	Val	Gln	Asn	Ser	Cys	Asp	Asn	
TGT	CAG	CCT	GGT	ACT	TTC	TGC	AGA	AAA	TAC	AAT	CCA	GTC	TGC	AAG	135
Cys	Gln	Pro	Gly	Thr	Phe	Cys	Arg	Lys	Tyr	Asn	Pro	Val	Cys	Lys	
H4-1BB FI															
AGC	TGC	CCT	CCA	AGT	ACC	TTC	TCC	AGC	ATA	GGT	GGA	CAG	CCG	AAC	180
Ser	Cys	Pro	Pro	Ser	Thr	Phe	Ser	Ser	Ile	Gly	Gly	Gln	Pro	Asn	
H4-1BB FII															
TGT	AAC	ATC	TGC	AGA	GTG	TGT	GCA	GGC	TAT	TTC	AGG	TTC	AAG	AAG	225
Cys	Asn	Ile	Cys	Arg	Val	Cys	Ala	Gly	Tyr	Phe	Arg	Phe	Lys	Lys	
TTT	TGC	TCC	TCT	ACC	CAC	AAC	GCG	GAG	TGT	GAG	TGC	ATT	GAA	GGA	270
Phe	Cys	Ser	Ser	Thr	His	Asn	Ala	Glu	Cys	Glu	Cys	Ile	Glu	Gly	
TTC	CAT	TGC	TTG	GGG	CCA	CAG	TGC	ACC	AGA	TGT	GAA	AAG	GAC	TGC	315
Phe	His	Cys	Leu	Gly	Pro	Gln	Cys	Thr	Arg	Cys	Glu	Lys	Asp	Cys	
AGG	CCT	GGC	CAG	GAG	CTA	ACG	AAG	CAG	GGT	TGC	AAA	ACC	TGT	AGC	360
Arg	Pro	Gly	Gln	Glu	Leu	Thr	Lys	Gln	Gly	Cys	Lys	Thr	Cys	Ser	
H4-1BB RI															
TTG	GGA	ACA	TTT	AAT	GAC	CAG	AAC	GGT	ACT	GGC	GTC	TGT	CGA	CCC	405
Leu	Gly	Thr	Phe	Asn	Asp	Gln	Asn	Gly	Thr	Gly	Val	Cys	Arg	Pro	
H4-1BB RII															
TGG	ACG	AAC	TGC	TCT	CTA	GAC	GGA	AGG	TCT	GTG	CTT	AAG	ACC	GGG	450
Trp	Thr	Asn	Cys	Ser	Leu	Asp	Gly	Arg	Ser	Val	Leu	Lys	Thr	Gly	
ACC	ACG	GAG	AAG	GAC	GTG	GTG	TGT	GGA	CCC	CCT	GTG	GTG	AGC	TTC	495
Thr	Thr	Glu	Lys	Asp	Val	Val	Cys	Gly	Pro	Pro	Val	Val	Ser	Phe	
TCT	CCC	AGT	ACC	ACC	ATT	TCT	GTG	ACT	CCA	GAG	GGA	GGA	CCA	GGA	540
Ser	Pro	Ser	Thr	Thr	Ile	Ser	Val	Thr	Pro	Glu	Gly	Gly	Pro	Gly	
GGG	CAC	TCC	TTG	CAG	GTC	CTT	ACC	TTG	TTC	CTG	GCG	CTG	ACA	TCG	585
Gly	His	Ser	Leu	Gln	Val	Leu	Thr	Leu	Phe	Leu	Ala	Leu	Thr	Ser	
GCT	TTG	CTG	CTG	GCC	CTG	ATC	TTC	ATT	ACT	CTC	CTG	TTC	TCT	GTG	630
Ala	Leu	Leu	Leu	Ala	Leu	Ile	Phe	Ile	Thr	Leu	Leu	Phe	Ser	Val	
CTC	AAA	TGG	ATC	AGG	AAA	AAA	TTC	CCC	CAC	ATA	TTC	AAG	CAA	CCA	675
Leu	Lys	Trp	Ile	Arg	Lys	Lys	Phe	Pro	His	Ile	Phe	Lys	Gln	Pro	
TTT	AAG	AAG	ACC	ACT	GGA	GCA	GCT	CAA	GAG	GAA	GAT	GCT	TGT	AGC	720
Phe	Lys	Lys	Thr	Thr	Gly	Ala	Ala	Gln	Glu	Glu	Asp	Ala	Cys	Ser	

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**Fig.1 cont'd**

TGC CGA TGT CCA CAG GAA GAA GAA GGA GGA GGA GGA GGC TAT GAG 765  
 Cys Arg Cys Pro Gln Glu Glu Glu Gly Gly Gly Gly Gly Tyr Glu

CTG TGA  
 Leu ---

771

TGTA	CTATCC	TAGGAGATGT	GTGGG	CCGAA	ACCGAGAAGC	ACTAGGACCC	821
CACCATCCTG	TGGAACAGCA	CAAGCAACCC	CACCACCCCTG	TTCTTACACA			871
TCATCCTAGA	TGATGTGTGG	GCGCGCACCT	CATCCAAGTC	TCTTCTAACG			921
CTAACATATT	TGTCTTTACC	TTTTTTAAAT	CTTTTTTTAA	ATTTAAATTT			971
TATGTGTGTG	AGTGTTTTGC	CTGCCTGTAT	GCACACGTGT	GTGTGTGTGT			1021
GTGTGTGACA	CTCCTGATGC	CTGAGGAGGT	CAGAAAGACAA	AGGGTTGGTT			1071
CCATAAGAAC	TGGAGTTATG	GATGGCTGTG	AGCCGGNNNG	ATAGGTCGGG			1121
ACGGAGACCT	GTCTTCTTAT	TTTAACGTGA	CTGTATAATA	AAAAAAAAT			1171
GATATTTCCG	GAATTGTAGA	GATTGTCTCTG	ACACCCTTCT	AGTTAATGAT			1221
CTAAGAGGAA	TTGTTGATAC	GTAGTATACT	GTAATAIGTGT	ATGTATATGT			1271
ATATGTATAT	ATAAGACTCT	TTTACTGTCA	AAGTCAACCT	AGAGTGTCTG			1321
GTTACCAAGT	CAATTTTATT	GGACATTTTA	CGTCACACAC	ACACACACAC			1371
ACACACACAC	ACGTTTATAC	TACGTACTGT	TATCGGTATT	CTACGTCATA			1421
TAATGGGATA	GGGTAAAAGG	AAACCAAAGA	GTGAGTGATA	TTATTGTGGA			1471
GGTGACAGAC	TACCCCTTCT	GGGTACGTAG	GGACAGACCT	CCTTCGGACT			1521
GTCTAAAAC	CCCCTTAGAA	GTCTCGTCAA	GTTCCCGGAC	GAAGAGGACA			1571
GAGGAGACAC	AGTCCGAAAA	GTTATTTTTC	CGGCMAATCC	TTTCCCTGTT			1621
TCGTGACACT	CCACCCCTTG	TGGACACTTG	AGTGTCTATCC	TTGCGCCGGA			1671
AGGTCAGGTG	GTACCCGTCT	GTAGGGGCGG	GGAGACAGAG	CCGCGGGGGA			1721
GCTACGAGAA	TCGACTCACA	GGGCGCCCCG	GGCTTCGCAA	ATGAAACTTT			1771
TTTAATCTCA	CAAGTTTCGT	CCGGGCTCGG	CGGACCTATG	GCGTCGATCC			1821
TTATTACCTT	ATCCTGGCGC	CAAGATAAAA	CAACCAAAAAG	CCTTGACTCC			1871
GGTACTAATT	CTCCCTGCCG	GCCCCCGTAA	GCATAACGCG	GCGATCTCCA			1921
CTTTAAGAAC	CTGGCCGCGT	TCTGCCTGGT	CTCGCTTTCG	TAAACGGTTC			1971
TTACAAAAGT	AATTAGTTCT	TGCTTTCAGC	CTCCAAGCTT	CTGCTAGTCT			2021
ATGGCAGCAT	CAAGGCTGGT	ATTTGCTACG	GCTGACCGCT	ACGCCGCCGC			2071
AATAAGGGTA	CTGGGCGGCC	CGTCGAAGGC	CCTTTGGTTT	CAGAAACCCA			2121
AGGCCCCCT	CATACCAACG	TTTCGACTTT	GATTCCTTGCC	GGTACGTGGT			2171
GGTGGGTGCC	TTAGCTCTTT	CTCGATAGTT	AGAC				2205

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*Fig. 2a*

human homologue of mouse 4-1bb

h4-1bb Length 838

```

1  AATCAGCTTT GCTAGTATCA TACCTGTGCC AGATTTTCATC ATGGGAAACA
51  GCTGTTACAA CATAGTAGCC ACTCTGTTGC TGGTCCTCAA CTTTGAGAGG
101 ACAAGATCAT TGCAGGATCC TTGTAGTAAC TGCCCAGCTG GTACATTCTG
151 TGATAATAAC AGGAATCAGA TTTGCAGTCC CTGTCCTCCA AATAGTTTCT
201 CCAGCGCAGG TGGACAAAGG ACCTGTGACA TATGCAGGCA GTGTAAAGGT
251 GTTTTCAGGA CCAGGAAGGA GTGTTCTCTC ACCAGCAATG CAGAGTGTGA
301 CTGCACTCCA GGGTTTCACT GCCTGGGGGC AGGATGCAGC ATGTGTGAAC
351 AGGATTGTAA ACAAGGTCAA GAACTGACAA AAAAAAGGTTG TAAAGACTGT
401 TGCTTTGGGA CATTTAACGA TCAGAAACGT GGCATCTGTC GACCCTGGAC
451 AAACGTGTTCT TTGGATGGAA AGTCTGTGCT TGTGAATGGG ACGAAGGAGA
501 GGGACGTGGT CTGTGGACCA TCTCCAGCTG ACCTCTCTCC GGGAGCATCC
551 TCTGTGACCC CGCCTGCCCC TCGGAGAGAG CCAGGACACT CTCCGCAGAT
601 CATCTCCTTC TTTCTTGCGC TGACGTGAC TGCCTTGCTC TTCCTGCTGT
651 TCTTCCTCAC GCTCCGTTTC TCTGTTGTTA AACGGGGCAG AAAGAACTC
701 CTGTATATAT TCAAACAACC ATTTATGAGA CCAGTACAAA CTACTCAAGA
751 GGAAGATGGC TGTAGCTGCC GATTTCCAGA AGAAGAAGAA GGAGGATGTG
801 AACTGTGAAA TGGAAATCAA TAGGGCTGTT GGGACTTT

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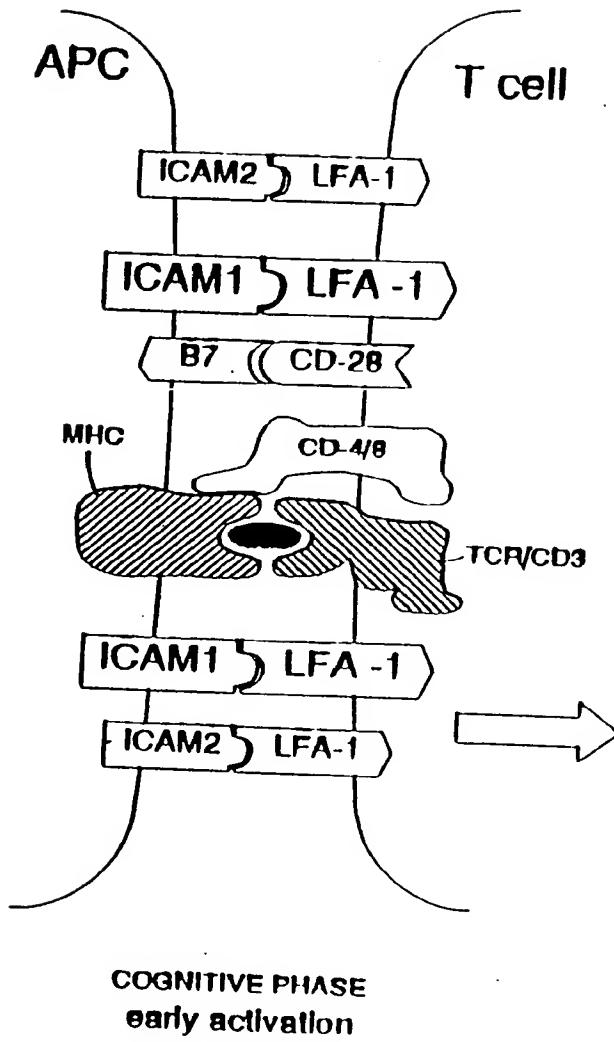
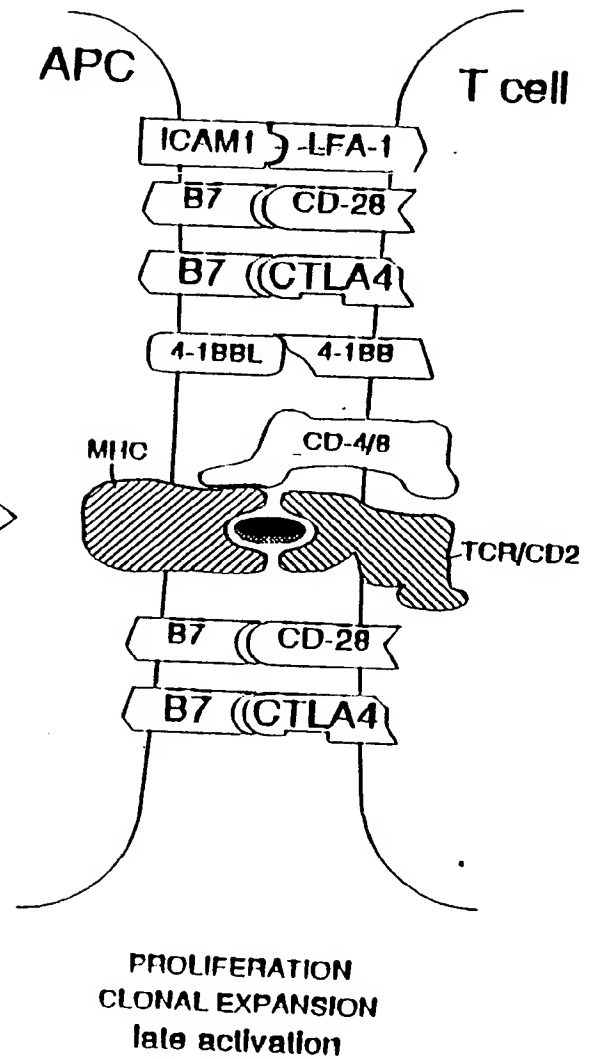
*Fig. 2b*

```

1  MGNSCYNIVA TLLLVLNFER TRSLQDFCSN CPAGTFCDNN RNQICSPCPP
51  NSFSSAGGQR TCDICRQCKG VERTRKECSS TSNAECDCTP GFHCLGAGCS
101 MCEQDCKQGQ ELTKKGCKDC CFGTFNDQKR GICRPWTNCS LDGKSVLVNG
151 TKERDVCVCGP SPADLSPGAS SVTPFAPARE FGHSPQIISF FLALTSTALL
201 FLLFFLTLRF SVVKRGRKKL LYIFKQPFMR PVQTTQEEDG CSCRFPEEEE
251 GGCEL

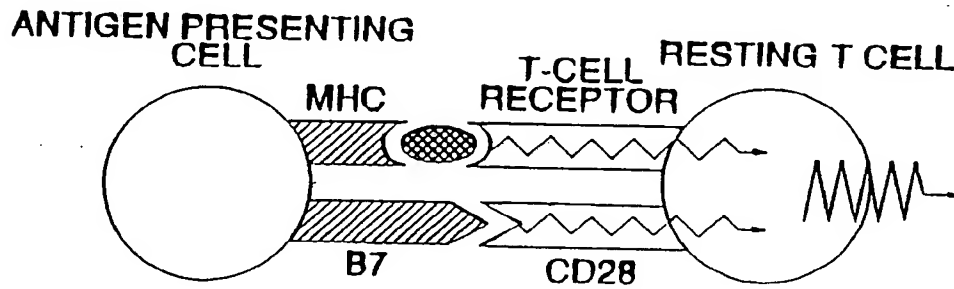
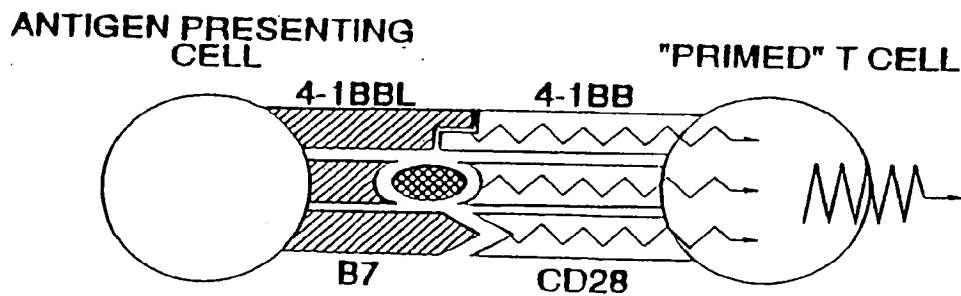
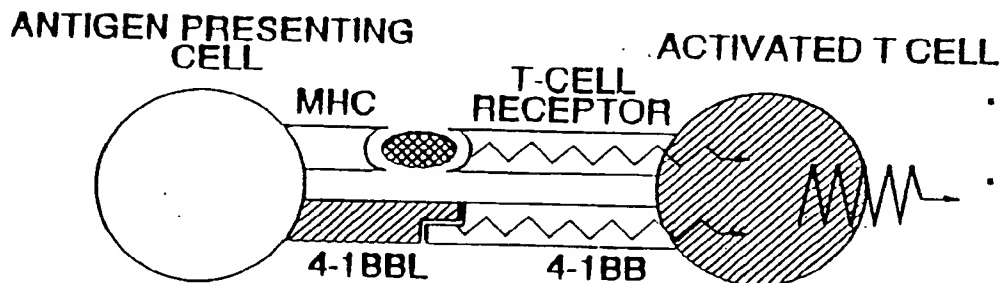
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*Fig. 3a**Fig. 3b*

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## NORMAL T-CELL ACTIVATION PATHWAY

*Fig. 4a**Fig. 4b**Fig. 4c*



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## BLOCKING STEPS IN T-CELL ACTIVATION PATHWAY

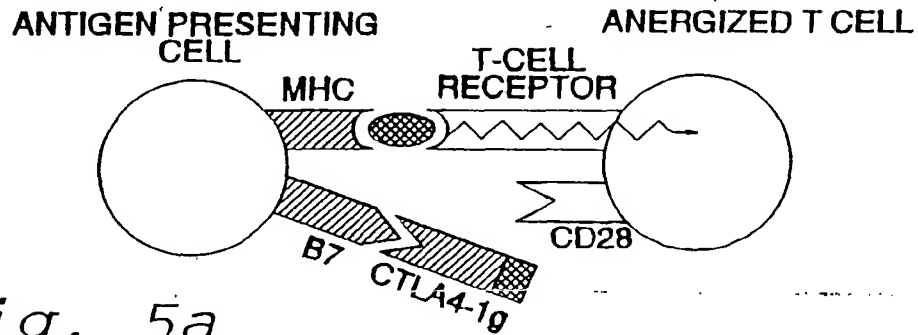


Fig. 5a

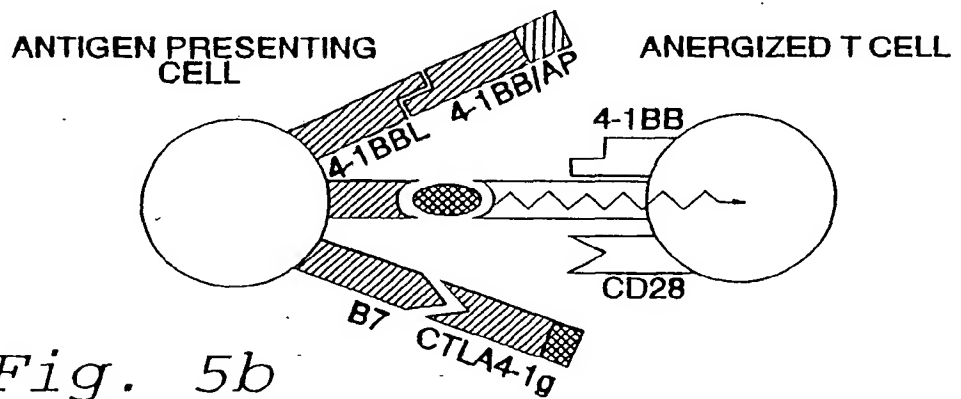


Fig. 5b

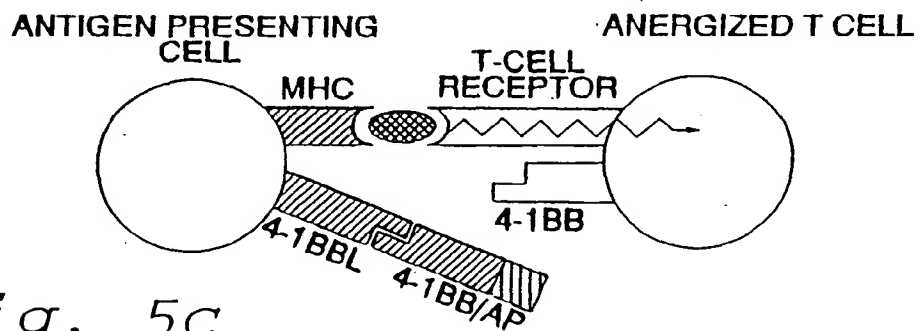


Fig. 5c